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(54) Title: METHOD FOR REDUCING LIGNING CONTENT IN PLANTS

(57) Abstract

The present invention relates to a method and reagent for reducing the lignin content in plants. Specifically, the invention entails the incorporation of an antisense gene for caffeic acid 3-O-methyltransferase into the genome of plants.

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METHOD FOR REDUCING LIGNIN CONTENT IN PLANTS

TECHNICAL FIELD OF THE INVENTION

This invention relates to a method for reducing the lignin content in plants using an antisense gene for caffeic acid 3-*O*-methyltransferase from alfalfa.

BACKGROUND OF THE INVENTION

Lignin is an insoluble polymer which occurs in the secondary thickening of plant cell walls and is primarily responsible for the rigidity of plant stems. Although lignin is essential for vascular function in plants, and may be involved in disease resistance in cereals, there is much interest in producing plants with reduced lignin content. Lignin residues are a problem in the paper processing industry. Further, the digestibility of forage grasses by cattle decreases with increasing lignin content. Lignin concentration has been reported to be the single most important measurable factor limiting the *in vitro* digestibility of other constituents including cellulose, hemicellulose, and neutral detergent fiber. Casler, "In vitro Digestibility of Dry Matter in Cell Wall Constituents of Smooth Bromegrass Forage," *Crop Sci.*, Vol. 27, pp. 931-934, 1987. Casler further reported that small increases (approximately 1%) in lignin content can result in relatively large decreases (approximately 7%) in digestibility of plant dry matter.

U.S. Patent No. 5,107,065 issued to Shewmaker, et al., on April 21, 1992, describes the regulation of gene expression in plant cells using antisense regulation. Antisense regulation involves the integration of a gene under the transcriptional control of a promoter which is functional in the host and in which the strand to be transcribed is complementary to the strand of DNA that is normally transcribed from the endogenous genes one wishes to regulate. The integrated gene is referred to as the antisense gene. U.S. Patent No. 5,107,065 describes methods and compositions for modulating RNA utilization, particularly modulation of a phenotypic property of a plant host cell. The patent discusses that the complementary sequence can be at least about 15

nucleotides in length, usually being fewer than about 5,000 nucleotides. According to the patent, the particular sites to which the sequence binds, and the length of the sequence, need to be determined based empirically on the experience observed with a particular sequence. Specifically, the patent describes the use of antisense technology in the regulated modulation of the expression of polygalacturonase in tomatoes. The patent describes that the ability to reduce the production of polygalacturonase could have a positive effect on the solids content of the tomato plant and improve tomato processing. Alteration of lignin content is discussed, and "loblolly pine, Douglas fir, and poplar, etc." are identified as potential targets and, more specifically, the "cinnamoyl alcohol-CoA: NADPH reductase or cinnamoyl alcohol dehydrogenase genes, etc." The patent does not disclose the reduction of lignin in plants using antisense technology, nor does it disclose the particular sequences, or their lengths, to be used.

Pillonel, et al., Involvement of Cinnamyl-Alcohol Dehydrogenase in the Control of Lignin Formation in *Sorghum bicolor* L. Moench," *Planta*, Vol. 185, pp. 538-544, 1991, attributed a major role in the regulation of the lignin content in a specific mutant of *Sorghum* demonstrating a 15-25% reduction in lignin concentration to depression of cinnamyl-alcohol dehydrogenase ("CAD") activity. Pillonel et al. reported a concomitant reduction in the product of the CAD enzyme, specifically, coniferyl alcohol. Contrastingly, an increase in ferulic acid, a product of the caffeic acid O-methyltransferase ("COMT") enzyme discussed below, was reported. The authors concluded that the structural modifications associated with the mutation were O-methyltransferase ("OMT") independent.

Canadian patent application No. 2,005,597 issued to Schuch, et al., on June 15, 1990, describes plants having reduced lignin, or a lignin of altered quality. According to the patent, recombinant DNA comprising a nucleotide sequence encoding mRNA which is substantially homologous or complementary to mRNA encoded by an endogenous plant gene, or part thereof, which encodes an enzyme essential to lignin biosynthesis is provided so that mRNA transcribed from the insert inhibits the production of the enzyme from the endogenous gene. Enzymes essential to lignin biosynthesis which were discussed include cinnamyl alcohol dehydrogenase (CAD), cinnamoyl-CoA reductase (CCR), and catechol-*O*-methyltransferase (synonymous with caffeic acid 3-*O*-methyltransferase, COMT). Specifically, antisense vectors for CAD were described. A reduction in CAD enzyme activity and an increase in neutral detergent fibre/cellulose values for transformed plants was provided. Although modification of lignin was claimed, no data reporting a reduction in lignin was provided.

In 1991, the purification and characterization of the caffeic acid 3-*O*-methyltransferase (COMT) enzyme from alfalfa was described. See Edwards, et al., "Purification and Characterization of *S* Adenosyl-L-Methionine:Caffeic Acid 3-*O*-Methyltransferase from suspension cultures of alfalfa (*Medicago sativa* L)," *Archives of Biochemistry and Biophysics*, Vol. 287, No. 2, pp. 372-379 (1991). Edwards, et al. reported that alfalfa plants had at least three O-methyltransferase (OMT) activities with distinct substrate specificities: 1) the 3-hydroxyl group of cinnamic acids, 2) the 2'-hydroxyl group of chalcones and 3) the 7-hydroxyl group of isoflavones. All enzymes had similar molecular weights of around 41,000 daltons. Edwards, et al. also reported that an antiserum raised against

purified COMT from aspen immunoprecipitated COMT from alfalfa.

In Gowri, et al., "Stress Responses in Alfalfa (*Medicago sativa* L), X. Molecular Cloning and Expression of *S*-Adenosyl-L-Methionine:Caffeic Acid 3-*O*-Methyltransferase, A Key Enzyme of Lignin Biosynthesis," *Plant Physiology*, Vol. 97, pp. 7-14, 1991 (incorporated herein by reference), a functionally active cDNA clone (pCOMT) encoding COMT was described. Gowri, et al. reported that the derived amino acid sequence of the enzyme from the cDNA is 86% identical to the COMT from aspen. Both strands of the cDNA had been sequenced from their ends and the sequence for the sense strand was reported.

Studies were undertaken to determine the possibility of perturbing lignin synthesis in plants by under-expression of COMT transcripts in alfalfa and other species using antisense RNA. Although lignin modification was discussed as being desirable in U.S. Patent No. 5,107,065, and claimed in Canadian Patent No. 2,005,597, neither publication demonstrates that an actual reduction in lignin was accomplished via the procedures described. As observed in U.S. Patent No. 5,107,065, the sites and length of sequences for effecting antisense suppression need to be determined empirically. In Cannon, et al., "Organ-Specific Modulation of Gene Expression in Transgenic Plants Using Antisense RNA," *Plant Molecular Biology*, Vol. 15, pp. 39-47, 1990, the authors discussed the ability to inhibit the *Escherichia coli* β-glucuronidase (GUS) gene expression in transgenic plants using a short 5′ sequence of the GUS messenger RNA. Cannon, et al. reported that the optimum size of an antisense RNA could be influenced by secondary structure at the N-terminal of a given mRNA. They also reported

that the size of the antisense RNA is likely to have a significant effect on the kinetics of hybridization. Successful antisense regulation was achieved in Cannon et al. with an antisense gene representing only a fragment of the complete endogenous gene. Similarly, in Sandler, et al., "Inhibition of Gene Expression in Transformed Plants by Antisense RNA," *Plant Molecular Biology*, Vol. 11, pp. 301-310, 1988, the authors reported that the most effective antisense sequences were those comprising less than the transcribed region, and reported that the suppressive sequences were derived from the 3′ half of the gene transcript. The gene involved was the nopaline synthase gene. Contrastingly, neither sequences upstream from the 3′ end, nor the entire transcribed region, appeared to be effective in suppressing the nopaline synthase gene.

Podila, et al. reported on the use of antisense technology in constructing transgenic tobacco plants utilizing antisense expression of an aspen xylem-specific O-methyltransferase (OMT). Podila, et al., "Antisense Expression of an Aspen O-methyltransferase Construct in Transgenic Tobacco via *Agrobacterium*", *Plant Physiology*, (supplement), Vol. 99, No. 1, p. 19, May 1992. Abnormal phenotype plants were described having a decreased OMT activity. The authors reported that the results indicated that the reduction of the level of a key enzyme in monolignol biosynthesis, namely OMT, does in some cases have an impact on plant development.

In summary, alteration of phenotypic properties in plants by antisense regulation of genes has been disclosed, and down-regulation of specific enzymes reportedly achieved. While the desirability of regulating lignin content of plants has been discussed, no one has heretofore demonstrated an actual reduction in

lignin content through the use of antisense technology. Thus, there has been a continuing need to provide a workable method for regulating lignin content in plants.

It has now been found that the lignin content of plants such as tobacco and alfalfa can be successfully lowered by utilizing an antisense gene for the enzyme caffeic acid 3-*O*-methyltransferase (COMT). By utilizing the technology disclosed herein, the phenotypic alteration of important forage plants can be achieved.

SUMMARY OF THE INVENTION

In one aspect, the present invention relates to a method for reducing the lignin content of plants comprising inserting a transcribable antisense gene for caffeic acid 3-*O*-methyltransferase of alfalfa into the genome of a plant.

In another aspect, the present invention relates to a reagent for transfecting plants to reduce the lignin content thereof comprising a transcribable antisense gene for caffeic acid 3-O-methyltransferase of alfalfa.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the vector construction strategy for tobacco transfection.

FIGURE 2 depicts the lignin content of transfected tobacco plants compared with controls.

FIGURE 3 is a southern blot of transgenic tobacco genomic DNA.

FIGURE 4 depicts the COMT activity of transfected tobacco plants and a control.

FIGURE 5 depicts the vector construction strategy for alfalfa transfection.

FIGURE 6 depicts the lignin content and COMT activity of transfected alfalfa plants compared with controls.

FIGURE 7a depicts the complementary sequence of the fragment (highlighted) of the gene utilized in the antisense vector construct for tobacco.

FIGURE 7b depicts the complementary sequence of the fragment (highlighted) of the gene utilized in the antisense vector construct for alfalfa.

FIGURE 8 depicts the lignin biosynthesis pathway.

DETAILED DESCRIPTION

Pillonel, et al. reported that plants exhibiting a reduction in lignin content exhibited a decrease in products of CAD. As can be seen in FIGURE 7, CAD (9) is involved near the end of the biosynthetic pathway for lignin. Therefore, modification of lignin content through down-regulation of CAD using antisense technology has been attempted. Contrastingly, Pillonel, et al. reported an increase in products of COMT involved earlier in the biosynthetic pathway of lignin (4). Nonetheless, once COMT cDNA was cloned, studies were undertaken to use antisense COMT constructs to modify the lignin content of plants. Inhibition of COMT blocks the formation of ferulic acid and should, thus, inhibit the synthesis of both coniferyl alcohol and sinapyl alcohol, the two major lignin monomers in dicotyledonous plants. This was borne out in the experiments and results reported upon here.

Transgenic tobacco and alfalfa plants which exhibited significantly reduced lignin content in young stems were successfully generated. Tobacco plants were initially chosen due to ease of manipulation (e.g., transformation and regeneration). The COMT cDNA (pCOMT derived from COMT mRNA) had been cloned from alfalfa previously (Gowri, et al., 1991). Its sequence was 77% and 74% identical to that of aspen and tobacco COMTs, respectively, and thus appears to be highly conserved among plants. Further, alfalfa COMT cDNA hybridized with tobacco DNA and RNA. This evidence suggested that it should be possible to inhibit lignin biosynthesis in tobacco by introducing an antisense alfalfa COMT gene into the plant.

In a preferred embodiment, the method according to the invention is carried out in forage plants. The same protocol could be applied to other forage legumes and

grasses, for which transformation/regeneration systems are available.

Example 1

Transfection of Tobacco Plants

1. **Vector construction**

FIGURE 1 shows the strategies used for vector construction. A 1.3 kb fragment of alfalfa COMT cDNA, pCOMT in FIGURE 1, was digested with various restriction enzymes, and released fragments were inserted into the vector pRTL2 in the orientation indicated by the arrows. The vector pRTL2 has a CaMV 35S promoter which can drive the expression of the inserted DNA fragment, but the vector is incapable of incorporating the DNA fragment into the targeted plant genome. The cassette containing the 35S promoter and the inserted DNA fragment was thus removed from pRTL2 by *Hin*dIII digestion and inserted into the binary vector pGA482 for insertion into the targeted plant genome. All elements between BL (border left) and BR (border right) in pGA482 can be inserted into the target plant genome by *Agrobacterium*-mediated transformation. Any suitable binary plant transformation vector can be used. The neomycin phosphotransferase (NPT II) segment in pGA482 was used as a selectable marker, since it confers kanamycin resistance on plants during transformation and regeneration. The construct derived from fragment B′ to B′, indicated with a thick arrow, was used in all the experiments for tobacco described below. This construct included an approximately 0.45 kb fragment of the antisense pCOMT gene. In FIGURES 1 and 5, "B" represents a *Bam*H1 restriction site and "B′" represents a *Bcl*I restriction site. The relevant restriction enzyme sites on the pCOMT sequence are depicted in FIGURE 7a. The portion of the pCOMT sequence used for the tobacco

construct is highlighted in FIGURE 7a and was essentially the sequence located between the two *BclI* restriction enzyme sites depicted in FIGURE 7a. Insertion of this portion of the sequence in inverted orientation into vector pRTL2 ensured transcription of the complementary, antisense strand. The orientation of the antisense gene was determined by restriction enzyme analysis using standard procedures as described by Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Lab, 2d ed., 1989, incorporated herein by reference.

2. Transformation and regeneration

Leaf disk transformation procedures (Rogers, et al., *Methods in Enzymology*, Vol. 118, pp. 627-640, 1986, incorporated herein by reference) were used for tobacco (*Nicotiana tabacum* cv. Xanthi) transformation. The binary vector pGA482 was mobilized into *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw method described by An, et al., Plant Molecular Biology Manual, Kluwer Academic Publishing, Netherlands, 1988 (incorporated herein by reference). A single colony of transformed bacteria was inoculated in Murashige and Skoog (MS) medium (Murashige and Skoog, *Physiologia Plantarum*, Vol. 15, pp. 473-497, 1962, incorporated herein by reference) and grown overnight. Sterilized young tobacco leaf disks were incubated in the bacterial culture and placed on MS medium containing kanamycin (100 ug/ml) and carbenicillin (500 ug/ml). Fifteen independent plants were regenerated from leaf disks; nine of those 15 were transferred to the greenhouse. The remaining six died before transferring. Seven plants were used for the further analyses discussed below.

Transgenic plants exhibited no morphological differences from control tobacco plants under normal

growth chamber and greenhouse conditions. Control tobacco plants were regenerated from leaf disks without transformation with foreign genes.

3. **Histochemical lignin estimation**

Cross sections of various parts of tobacco stems were taken with razor blades, soaked in ethanol for at least 30 minutes, and stained with a drop of 0.5% phloroglucinol (prepared in 50% HCl). After 5 minutes of staining, the section was observed with a microscope and the image photographed with an attached camera. Young stems exhibited much lower lignin content in transformed plants than in control plants.

4. **Soluble phenolic analyses**

Alteration in the lignin biosynthetic pathway may also change the content and composition of soluble phenolic compounds (e.g., decrease metabolites derived from the COMT reaction). Phenolic analysis was performed as described in Elkind, et al., *Proc. Natl. Acad. Sci. USA*, Vol. 87, pp. 9057-9061, 1990 (incorporated herein by reference). Tobacco stems were cut in half and upper portions were homogenized with a mortar and pestle, extracted with water once, and then extracted with methanol for at least two days with several changes of methanol. After extraction, debris (cell wall material) was saved for lignin analysis (see below). Water and methanol fractions were combined and methanol was evaporated by blowing a stream of nitrogen gas through the samples. Chlorophylls and other pigments were removed by partitioning the aqueous phase against hexane. Total soluble phenolics were finally obtained by passing the aqueous phases through C18 SEP-PAK cartridges (Waters, Milford, MA). Concentrated phenolic extracts were

analyzed by high pressure liquid chromatography (HPLC) using the method of Elkind, et al. (1990). The transformed tobacco plant chromatograph had one peak which was much lower than that of the same retention time in the chromatograph of the control. There were no other significant changes observed, and that peak has not been identified.

5. Total lignin analysis (thioglycolic acid method)

Cell wall material derived from methanol extraction (see Soluble phenolic analyses) was used for lignin estimation by the method of Doster, et al. *Phytopathology*, Vol. 78, pp. 473-477, 1988, incorporated herein by reference. Dried cell wall material (0.1 gm) was incubated in 5 ml 2N HCl containing 10% (v/v) thioglycolic acid at 95°C for 2 hours. After centrifugation, the acidic supernatant was removed and the pellet was suspended in 5 ml of 0.5 N NaOH for at least 20 hours with gentle shaking. Debris was removed by centrifugation and the thioglycolic acid-lignin complex was re-precipitated by adding 1 ml of concentrated HCl to the alkaline supernatant. Pellets were resuspended in 2 ml 0.1 N NaOH. The absorbance of the complex at 280 nm was used to calculate the lignin content in control and transgenic tobacco stems. The results are depicted in FIGURE 2. The average lignin content in the transgenic plants was 20-50% lower than the average of the control plants. Although transgenic plant B107 exhibits a higher lignin content than the other transgenic plants, it was later determined that plant B107 did not contain the antisense gene fragment. See FIGURE 3.

6. Lignin composition analysis (nitrobenzene oxidation method)

Thioglycolic acid reaction products were used for nitrobenzene oxidation and HPLC analysis as described previously (Pillonel, et al., *Planta*, Vol. 185, pp. 538-544, 1991, incorporated herein by reference). One ml of the reaction product was mixed with 5 ml 2 M NaOH containing 100 ul of nitrobenzene in an acid reaction bomb. The bomb was incubated at 165°C in a glycerol bath for 2 hours. The reaction products were acidified to pH 3 and partitioned against CH_2Cl_2. The organic phase was dried in a heat block (at about 50°C) by passing a stream of nitrogen gas overwards. The residue was dissolved in methanol and analyzed by HPLC. The results indicated that the lignin composition was similar in the controls and the transgenic plants. There was no major alteration in relative levels of the components (i.e., sinapyl, coniferyl, and coumaryl units, see FIGURE 8).

7. COMT Activity Analysis

COMT was assayed according to Edwards, et al., *Arch. Biochem, Biophys.*, vol. 287, pp. 372-379, 1991, incorporated herein by reference. Plant tissues were homogenized in 100 mM Tris-HCl, pH 7.5, 2 mM EDTA and 5 mM dithiothreitol. The assay reaction was initiated by adding the substrates caffeic acid and [methyl-^{14}C]S-adenosyl-L-methionine to 150 μl of plant extract, and the reaction mixture was incubated at 37°C for 30 minutes. The assays were stopped with 30 μl of 1 N HCl, and labeled products were partitioned into 250 μl of ethyl acetate: hexane (1:1, v/v). After centrifugation, 150 μl of the organic phase was analyzed by scintillation counting. The results are depicted in FIGURE 4. Six of the seven transgenic plants tested exhibited from 20% to 60% of the

average control value. The control value is the average of three independent untransformed plants plus plant B107, which was transformed but does not contain the antisense gene (see FIGURE 3).

8. Nucleic acid analysis

The presence of the COMT antisense construct in transgenic plants was determined by Southern blot analysis. Total DNA isolated from tobacco leaves (Junghans, et al., *Biotechniques*, Vol. 8, p. 176, 1990, incorporated herein by reference) was digested with the restriction enzyme *Hin*dIII, resolved on an agarose gel and blotted onto a nylon membrane. Blot hybridization and other standard manipulations were as described by Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Lab, 2d ed., 1989, incorporated herein by reference. The results are depicted in FIGURE 3. All transgenic plants except B107 contained an approximately 1.4 kb fragment hybridizing to the pCOMT sequence.

A COMT antisense construct was successfully introduced into transgenic tobacco plants. All of the seven transgenic plants analyzed showed NPT II activity indicating that these plants were truly transformed with the construct. Six of the seven plants exhibited various ranges of COMT enzyme activities, which were 20 to 60% of the COMT activities in control plants. Histochemical staining for lignin in the stems of the transgenic plants revealed a significant reduction of lignin in six of the seven transgenic plants. Southern blot analysis (FIGURE 3) indicated that the transgenic plant (B107) with normal lignin content (compared to control plants) did not contain the COMT antisense fragment. These results indicated that the reduction in lignin content was not due to plant structural or other alterations during

transformation and/or regeneration. This has been confirmed by the analysis of progeny plants obtained by self fertilization of the primary transformants. Such plants harboring the COMT antisense gene again show reduction in lignin content. Data from total lignin analysis confirmed the observations from histochemical staining. Alteration of the lignin biosynthetic pathway apparently did not change the lignin composition, although the HPLC profile of soluble phenolics was modified somewhat. It is likely that increases in the ratios of coumaryl alcohol to coniferyl/sinapyl alcohols in lignin would reduce forage digestibility.

The overall results confirmed that production of lignin in tobacco plants can be down-regulated by introducing an antisense gene of the alfalfa COMT enzyme in the lignin biosynthetic pathway.

Example 2
Transfection of Alfalfa Plants

Alfalfa transformation and regeneration

Alfalfa (*Medicago sativa* L.cv Regen SY) trifoliate leaves from greenhouse plants were sterilized with soapy water, 70% ethanol, and 20% Clorox plus 0.1% SDS, and washed three times with sterile water. Each leaf was cut into a square and dropped into a suspension of transformed *Agrobacterium* with the COMT antisense construct as described below. The inoculated alfalfa leaf disks were removed from the bacterial suspension and placed on B5h medium. After three days incubation, the leaf disks were rinsed with sterile water to remove excess *Agrobacteria* and placed on B5h medium containing 25 mM kanamycin and 250 mM carbenicillin. One week after embryos had formed, the leaf disks were transferred to B5h medium minus growth

regulators. When embryos were large enough to begin rooting, the leaf disks were transferred to Bio2y medium containing 25 mM kanamycin and 250 mM carbenicillin, and finally transferred to MS medium after sufficient roots had developed. Cell culture media (B5h and Bio2y) are described in Atanassov, et al., *Plant Cell, Tissue and Organ Culture*, Vol. 3, pp. 149-162, 1984, incorporated herein by reference.

The COMT antisense construct was prepared as in Example 1 but using a different fragment of the pCOMT sequence. A 0.6 kb fragment of the alfalfa pCOMT sequence (from the 5′ end to the B′ site, marked with a thick arrow in FIGURE 5) was used. The relevant restriction enzyme sites on the pCOMT sequence are depicted in FIGURE 7b. The portion of the pCOMT sequence used for the alfalfa construct is highlighted in FIGURE 7b and is essentially the sequence located between the *Bam*HI and the leftmost *Bcl*I restriction enzyme sites depicted. Insertion of this portion of the sequence in inverted orientation into vector pRTL2 ensured transcription of the complementary, antisense strand. Thirty four transgenic alfalfa plants were generated which all exhibited NPT II activity. Control plants were taken through the full regeneration protocol.

<u>COMT activity assay</u>

COMT activity was determined as described for tobacco. The results are depicted in FIGURE 6. COMT activity in antisense plants averaged approximately 50% of that of the control. The values for the control are the average and spread of values (as indicated by the bars) from four independent control plants.

Total lignin estimation

The thioglycolic acid method was used for lignin estimation as described for tobacco. Results of COMT activity assay and lignin estimation for transgenic alfalfa are shown in FIGURE 6. These data are for the upper parts of whole seedlings (including leaves). Lignin content averaged approximately 80% of the control. No direct correlation between lignin content and COMT activity was observed. However, 14 of the 34 transgenic plants exhibited both lower COMT activity (35-80%) and lignin content (72-94%) as compared with controls.

The above results indicate that incorporation of the COMT antisense construct into alfalfa plants can lead to both reduced COMT activity and reduced lignin content.

The above examples were meant to be illustrative of the invention and were not meant to limit the invention in any way. It will be apparent to those skilled in the art that modifications can be made which would still be within the scope of the invention.

SEQUENCE LISTING

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(B) TELEFAX: 214/939-4600

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1341 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Alfalfa
(B) STRAIN: Medicago Sativa L

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: lambda ZAP II cDNA
(B) CLONE: Clone pCOMT1

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (541..1016)
(D) OTHER INFORMATION: /note= "The inverted complement of this portion was the antisense fragment used in the tobacco vector construct."

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(A) NAME/KEY: misc_feature
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      20                  25                  30

ATG ATT TTG AAA TCA GCT CTT GAA CTT GAT CTC TTA GAA ATC ATT GCT       201
Met Ile Leu Lys Ser Ala Leu Glu Leu Asp Leu Leu Glu Ile Ile Ala
  35                  40                  45
```

```
AAA GCT GGA CCT GGT GCT CAA ATT TCA CCT ATT GAA ATT GCT TCT CAG        249
Lys Ala Gly Pro Gly Ala Gln Ile Ser Pro Ile Glu Ile Ala Ser Gln
 50                  55                  60                  65

CTA CCA ACA ACT AAC CCT GAT GCA CCA GTT ATG TTG GAC CGA ATG TTG        297
Leu Pro Thr Thr Asn Pro Asp Ala Pro Val Met Leu Asp Arg Met Leu
                70                  75                  80

CGT CTC TTG GCT TGT TAC ATA ATC CTC ACA TGT TCA GTT CGT ACT CAA        345
Arg Leu Leu Ala Cys Tyr Ile Ile Leu Thr Cys Ser Val Arg Thr Gln
            85                  90                  95

CAA GAT GGA AAG GTT CAG AGA CTT TAT GGT TTG GCT ACT GTT GCT AAG        393
Gln Asp Gly Lys Val Gln Arg Leu Tyr Gly Leu Ala Thr Val Ala Lys
        100                 105                 110

TAT TTG GTT AAG AAT GAA GAT GGT GTA TCC ATT TCT GCT CTT AAT CTC        441
Tyr Leu Val Lys Asn Glu Asp Gly Val Ser Ile Ser Ala Leu Asn Leu
    115                 120                 125

ATG AAT CAG GAT AAA GTG CTC ATG GAA AGC TGG TAC CAC CTA AAA GAT        489
Met Asn Gln Asp Lys Val Leu Met Glu Ser Trp Tyr His Leu Lys Asp
130                 135                 140                 145

GCA GTC CTT GAT GGG GGC ATT CCA TTC AAC AAG GCT TAT GGA ATG ACA        537
Ala Val Leu Asp Gly Gly Ile Pro Phe Asn Lys Ala Tyr Gly Met Thr
                150                 155                 160

GCC TTT GAA TAC CAT GGA ACA GAT CCA AGG TTT AAC AAG GTT TTC AAC        585
Ala Phe Glu Tyr His Gly Thr Asp Pro Arg Phe Asn Lys Val Phe Asn
            165                 170                 175

AAG GGG ATG TCT GAT CAC TCT ACC ATC ACA ATG AAG AAA ATT CTT GAG        633
Lys Gly Met Ser Asp His Ser Thr Ile Thr Met Lys Lys Ile Leu Glu
        180                 185                 190

ACC TAC ACA GGT TTT GAA GGC CTT AAA TCT CTT GTT GAT GTA GGT GGT        681
Thr Tyr Thr Gly Phe Glu Gly Leu Lys Ser Leu Val Asp Val Gly Gly
    195                 200                 205

GGT ACT GGA GCT GTA ATT AAC ACG ATT GTC TCA AAA TAT CCC ACT ATA        729
Gly Thr Gly Ala Val Ile Asn Thr Ile Val Ser Lys Tyr Pro Thr Ile
210                 215                 220                 225

AAG GGT ATA AAT TTT GAT TTA CCC CAT GTC ATT GAA GAT GCT CCA TCT        777
Lys Gly Ile Asn Phe Asp Leu Pro His Val Ile Glu Asp Ala Pro Ser
                230                 235                 240

TAT CCA GGA GTT GAG CAT GTT GGT GGA GAC ATG TTT GTC AGT ATT CCA        825
Tyr Pro Gly Val Glu His Val Gly Gly Asp Met Phe Val Ser Ile Pro
            245                 250                 255

AAG GCT GAT GCT GTT TTT ATG AAG TGG ATT TGT CAT GAC TGG AGT GAT        873
Lys Ala Asp Ala Val Phe Met Lys Trp Ile Cys His Asp Trp Ser Asp
        260                 265                 270

GAG CAC TGC TTG AAA TTT TTG AAG AAC TGC TAT GAG GCA CTG CCA GAC        921
Glu His Cys Leu Lys Phe Leu Lys Asn Cys Tyr Glu Ala Leu Pro Asp
    275                 280                 285
```

```
AAT GGA AAA GTG ATT GTG GCA GAA TGC ATA CTT CCA GTG GCT CCA GAT        969
Asn Gly Lys Val Ile Val Ala Glu Cys Ile Leu Pro Val Ala Pro Asp
290                 295                 300                 305

TCA AGC CTG GCC ACA AAA GGT GTG GTT CAC ATT GAT GTG ATC ATG TTG       1017
Ser Ser Leu Ala Thr Lys Gly Val Val His Ile Asp Val Ile Met Leu
                310                 315                 320

GCT CAT AAT CCT GGT GGG AAA GAG AGA ACA CAA AAA GAG TTT GAG GAT       1065
Ala His Asn Pro Gly Gly Lys Glu Arg Thr Gln Lys Glu Phe Glu Asp
            325                 330                 335

CTT GCC AAA GGT GCT GGA TTC CAA GGT TTC AAA GTC CAT TGT AAT GCT       1113
Leu Ala Lys Gly Ala Gly Phe Gln Gly Phe Lys Val His Cys Asn Ala
        340                 345                 350

TTC AAC ACA TAC ATC ATG GAG TTT CTT AAG AAG GTT TAATTTCTTT            1159
Phe Asn Thr Tyr Ile Met Glu Phe Leu Lys Lys Val
    355                 360                 365

GGTGTGTTGC ATCTGAGTTT TGATATTGAG ATTGTGGTTG TGCTTCTACT TACCTAAGCT     1219

TTCCCCATAA AAATATGTGA TTTCCACTTC TATTCGGTAG GAAAATAATA ATGAGAAAGT     1279

TCATTGTAAT ATTGCCTATA TAAATGAACA TTGTTTCATA TTGTGGATTA TAAAAAAAAA     1339

AA                                                                    1341
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 365 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Gly Ser Thr Gly Glu Thr Gln Ile Thr Pro Thr His Ile Ser Asp
 1               5                  10                  15

Glu Glu Ala Asn Leu Phe Ala Met Gln Leu Ala Ser Ala Ser Val Leu
            20                  25                  30

Pro Met Ile Leu Lys Ser Ala Leu Glu Leu Asp Leu Leu Glu Ile Ile
        35                  40                  45

Ala Lys Ala Gly Pro Gly Ala Gln Ile Ser Pro Ile Glu Ile Ala Ser
    50                  55                  60

Gln Leu Pro Thr Thr Asn Pro Asp Ala Pro Val Met Leu Asp Arg Met
65                  70                  75                  80

Leu Arg Leu Leu Ala Cys Tyr Ile Ile Leu Thr Cys Ser Val Arg Thr
                85                  90                  95

Gln Gln Asp Gly Lys Val Gln Arg Leu Tyr Gly Leu Ala Thr Val Ala
            100                 105                 110
```

```
Lys Tyr Leu Val Lys Asn Glu Asp Gly Val Ser Ile Ser Ala Leu Asn
        115                 120                 125

Leu Met Asn Gln Asp Lys Val Leu Met Glu Ser Trp Tyr His Leu Lys
    130                 135                 140

Asp Ala Val Leu Asp Gly Gly Ile Pro Phe Asn Lys Ala Tyr Gly Met
145                 150                 155                 160

Thr Ala Phe Glu Tyr His Gly Thr Asp Pro Arg Phe Asn Lys Val Phe
                165                 170                 175

Asn Lys Gly Met Ser Asp His Ser Thr Ile Thr Met Lys Lys Ile Leu
            180                 185                 190

Glu Thr Tyr Thr Gly Phe Glu Gly Leu Lys Ser Leu Val Asp Val Gly
        195                 200                 205

Gly Gly Thr Gly Ala Val Ile Asn Thr Ile Val Ser Lys Tyr Pro Thr
    210                 215                 220

Ile Lys Gly Ile Asn Phe Asp Leu Pro His Val Ile Glu Asp Ala Pro
225                 230                 235                 240

Ser Tyr Pro Gly Val Glu His Val Gly Gly Asp Met Phe Val Ser Ile
                245                 250                 255

Pro Lys Ala Asp Ala Val Phe Met Lys Trp Ile Cys His Asp Trp Ser
            260                 265                 270

Asp Glu His Cys Leu Lys Phe Leu Lys Asn Cys Tyr Glu Ala Leu Pro
        275                 280                 285

Asp Asn Gly Lys Val Ile Val Ala Glu Cys Ile Leu Pro Val Ala Pro
    290                 295                 300

Asp Ser Ser Leu Ala Thr Lys Gly Val Val His Ile Asp Val Ile Met
305                 310                 315                 320

Leu Ala His Asn Pro Gly Gly Lys Glu Arg Thr Gln Lys Glu Phe Glu
                325                 330                 335

Asp Leu Ala Lys Gly Ala Gly Phe Gln Gly Phe Lys Val His Cys Asn
            340                 345                 350

Ala Phe Asn Thr Tyr Ile Met Glu Phe Leu Lys Lys Val
        355                 360                 365
```

WE CLAIM:

1. A method for reducing the lignin content of plants comprising:

a) transfecting plant cells with a vector comprising a transcribable antisense gene for a caffeic acid 3-*O*-methyltransferase gene of alfalfa, or portions thereof;

b) growing said transfected cells under conditions conducive to regeneration and mature plant growth;

c) screening mature plants for lignin content; and

d) selecting mature plants having a decreased lignin content as compared with controls for propagation.

2. The method of Claim 1 wherein the transcribable antisense gene is about 0.45 kb and is complementary to a sequence of about 0.45 kb of the caffeic acid 3-*O*-methyltransferase gene of alfalfa as depicted in FIGURE 7a.

3. The method of Claim 1 wherein the transcribable antisense gene is about 0.6 kb and is complementary to a sequence of about 0.6 kb of the caffeic acid 3-*O*-methyltransferase gene of alfalfa as depicted in FIGURE 7b.

4. The method of Claim 1 wherein the vector further comprises:

a 35S promotor, a termination codon, and a kanamycin resistance marker.

5. A reagent for use in reducing the lignin content in plants comprising:

a transcribable antisense gene for a caffeic acid 3-*O*-methyltransferase gene of alfalfa, or portions thereof.

6. The reagent of Claim 5 wherein the transcribable antisense gene is about 0.45 kb and is complementary to a sequence of about 0.45 kb of the caffeic acid 3-*O*-methyltransferase gene of alfalfa as depicted in FIGURE 7a.

7. The reagent of Claim 5 wherein the transcribable antisense gene is about 0.6 kb and is complementary to a sequence of about 0.6 kb of the caffeic acid 3-*O*-methyltransferase gene of alfalfa as depicted in FIGURE 7b.

8. The reagent of Claim 5 further comprising a 35S promoter, a termination codon, and a kanamycin resistance marker.

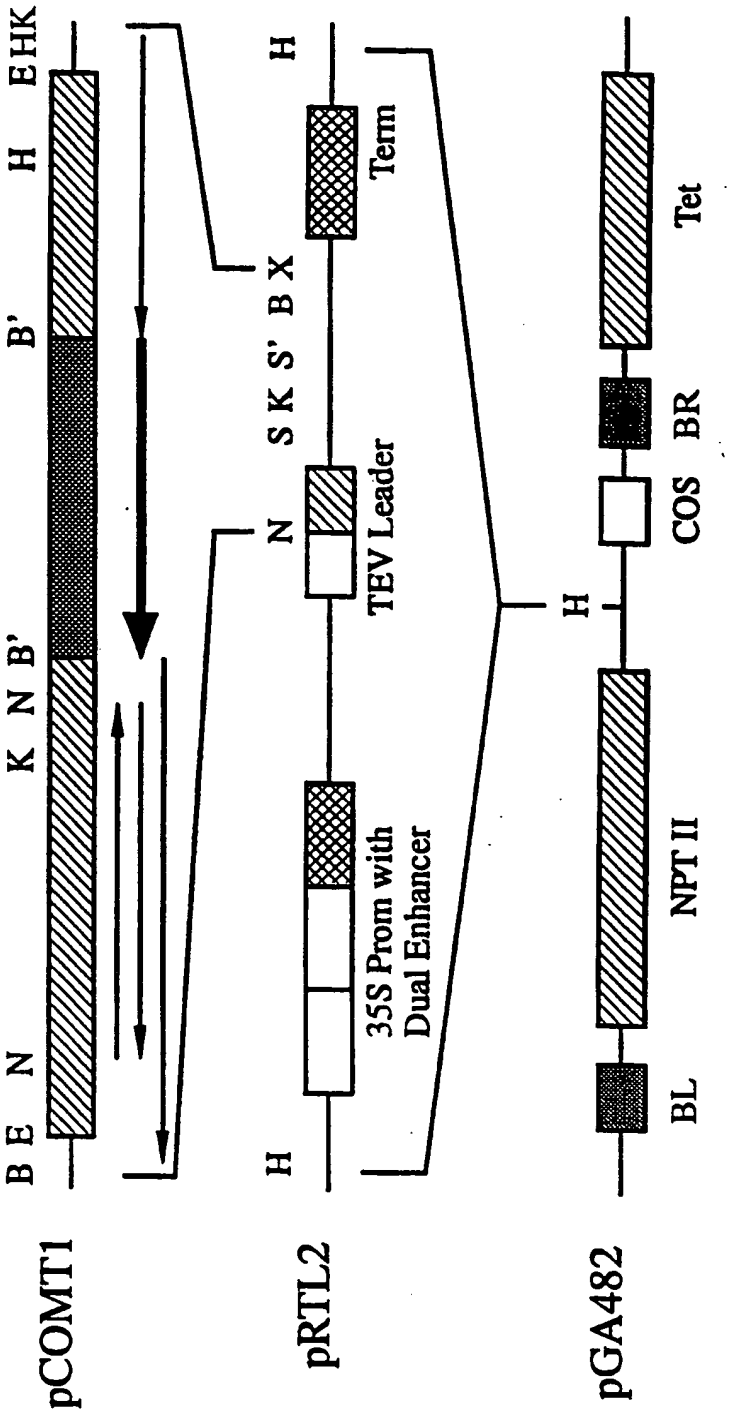
B E N
K N B'
B'
H E H K
pCOMT1
H
N
S K S' B X
TEV Leader
35S Prom with
Dual Enhancer
H
Term
pRTL2
H
COS
BR
Tet
BL
NPT II
pGA482

FIGURE 1

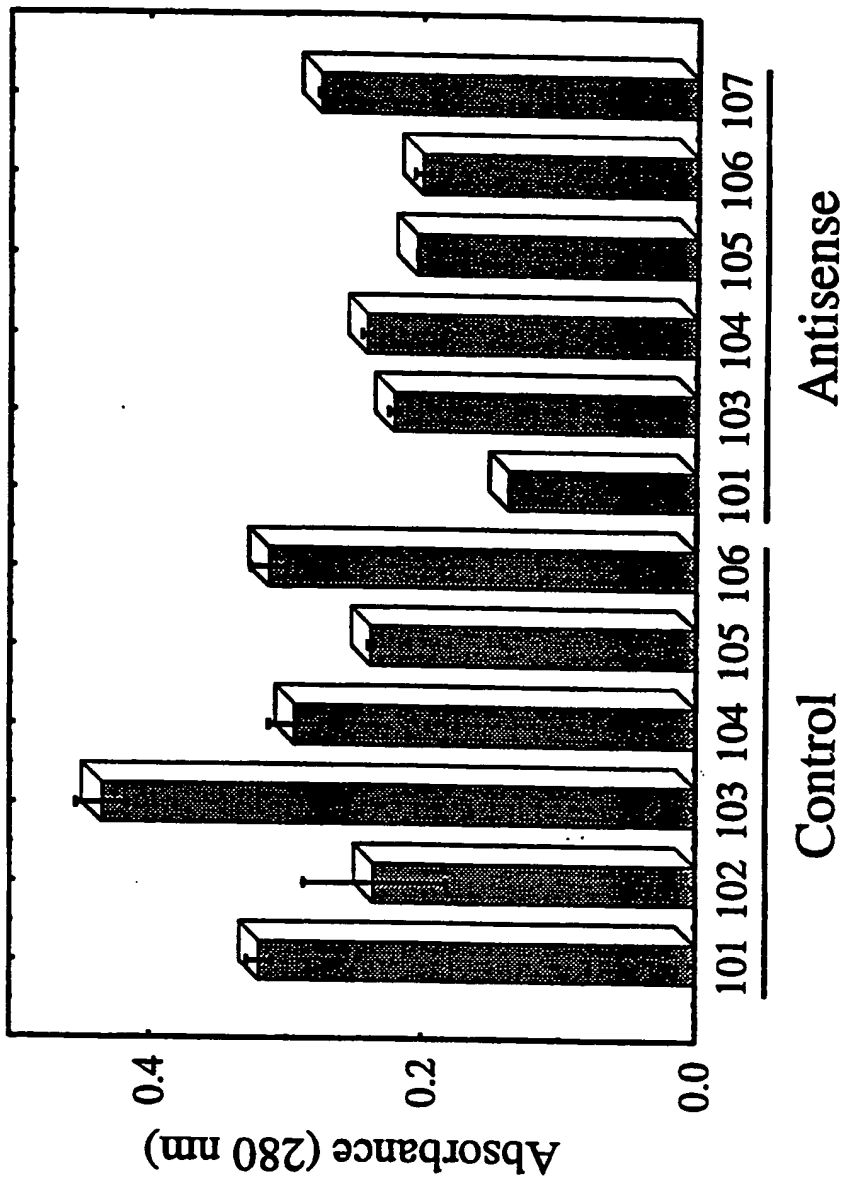
Absorbance (280 nm)
0.0
0.2
0.4
101 102 103 104 105 106
Control
101 103 104 105 106 107
Antisense

FIGURE 2

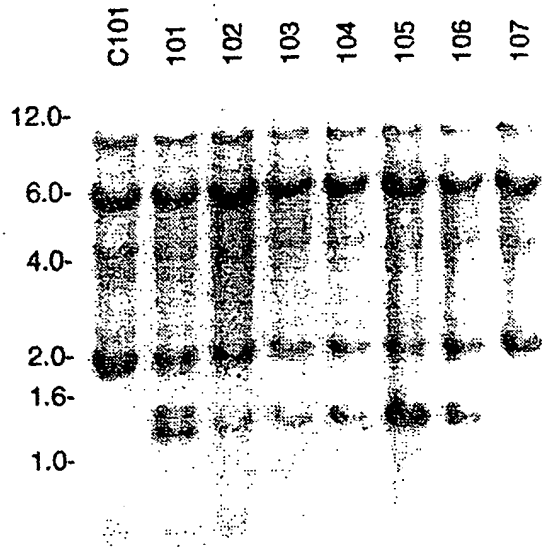

FIGURE 3

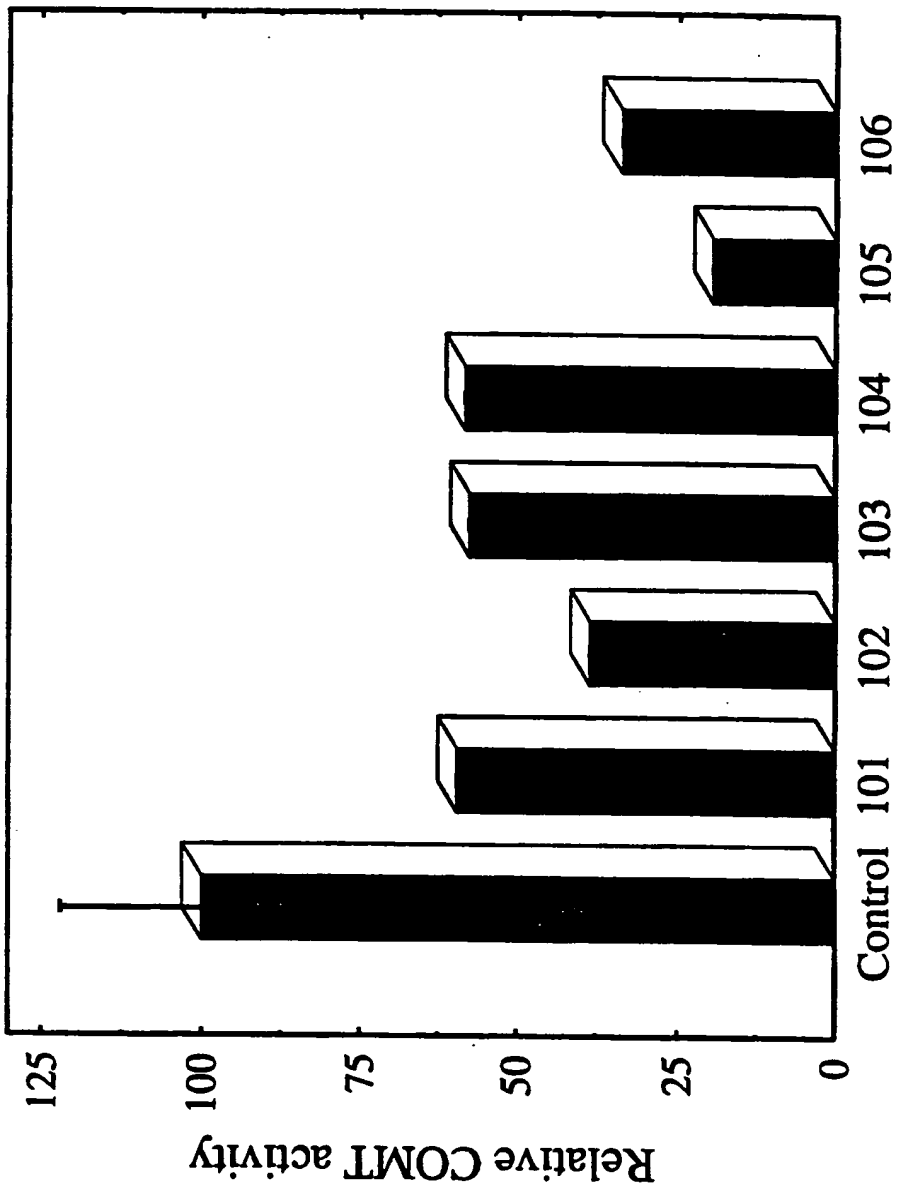
Relative COMT activity
125
100
75
50
25
0
Control
101
102
103
104
105
106

FIGURE 4

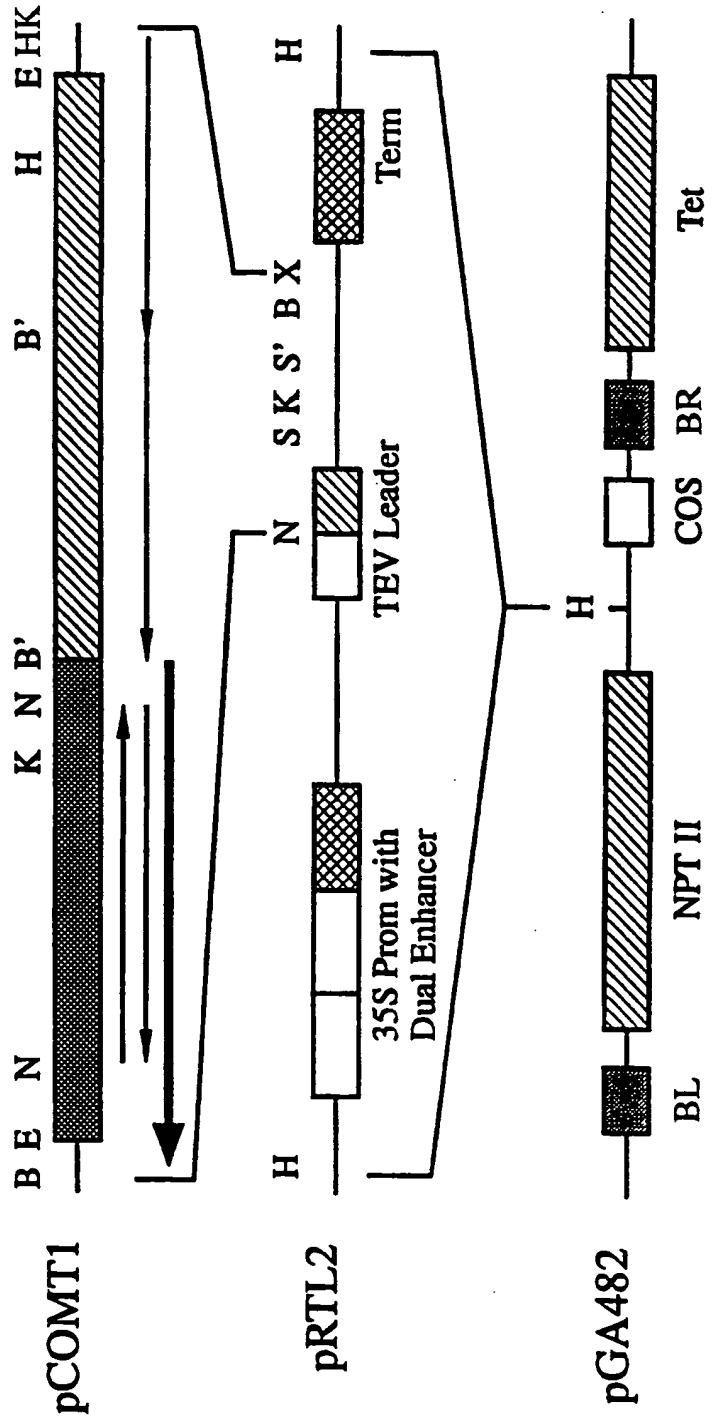
B E N
K N B'
B' H E H K
pCOMT1
H
35S Prom with Dual Enhancer
N
TEV Leader
S K S' B X
H
Term
pRTL2
H
BL
NPT II
COS
BR
Tet
pGA482

FIGURE 5

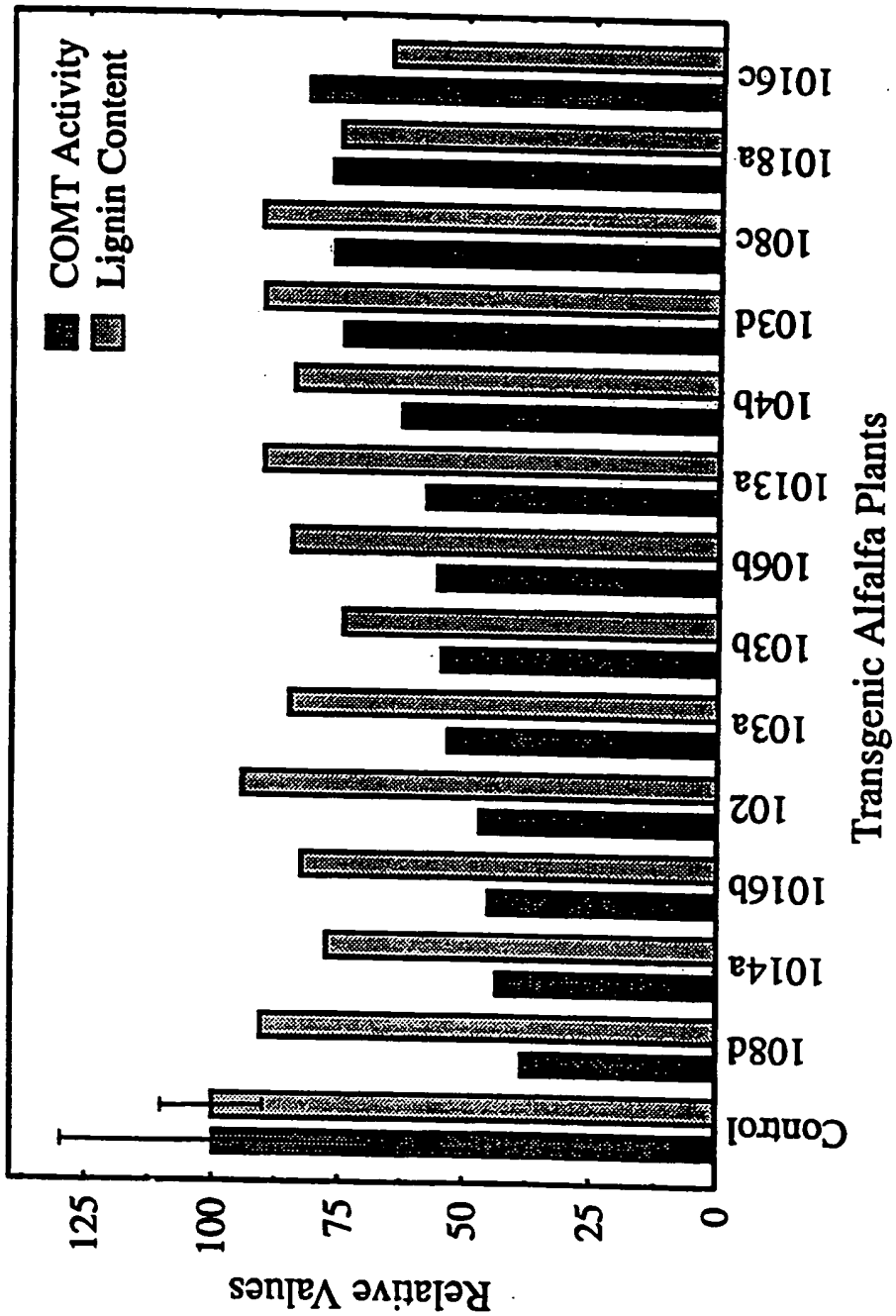
COMT Activity
Lignin Content
Relative Values
0
25
50
75
100
125
Control
108d
101 4a
101 6b
102
103a
103b
106b
101 3a
104b
103d
108c
101 8a
101 6c
Transgenic Alfalfa Plants

FIGURE 6

COMT sequence starts from here

```
   1  ggatcccccgggctgcaggaattcAATCTCACAAAAACCTCATCAATCACAACCATGGGT  60
  61  TCAACAGGTGAAACTCAAATAACACCAACCCACATATCAGATGAAGAAGCAAACCTCTTC 120
 121  GCCATGCAACTAGCAAGTGCTTCAGTTCTTCCCATGATTTTGAAATCAGCTCTTGAACTT 180
 181  GATCTCTTAGAAATCATTGCTAAAGCTGGACCTGGTGCTCAAATTTCACCTATTGAAATT 240
 241  GCTTCTCAGCTACCAACAACTAACCCTGATGCACCAGTTATGTTGGACCGAATGTTGCGT 300
 301  CTCTTGGCTTGTTACATAATCCTCACATGTTCAGTTCGTACTCAACAAGATGGAAAGGTT 360
 361  CAGAGACTTTATGGTTTGGCTACTGTTGCTAAGTATTTGGTTAAGAATGAAGATGGTGTA 420
 421  TCCATTTCTGCTCTTAATCTCATGAATCAGGATAAAGTGCTCATGGAAAGCTGGTACCAC 480
 481  CTAAAAGATGCAGTCCTTGATGGGGGCATTCCATTCAACAAGGCTTATGGAATGACAGCC 540
                                                        B
                                                        c
                                                        1
                                                        I
 541  TTTGAATACCATGGAACAGATCCAAGGTTTAACAAGGTTTTCAACAAGGGGATGTCTGAT 600
 601  CACTCTACCATCACAATGAAGAAAATTCTTGAGACCTACACAGGTTTTGAAGGCCTTAAA 660
 661  TCTCTTGTTCATGTACGTGGTGGTACTGGAGCTGTAATTAACACGATTGTCTCAAAATAT 720
 721  CCCACTATAAAGGGTATAAATTTTGATTTACCCCATCTCATTCAAGATGCTCCATCTTAT 780
 781  CCAGGAGTTGAGCATGTTGGTGGAGACATGTTTGTCAGTATTCCAAAGGCTGATGCTGTT 840
 841  TTTATGAAGTGGATTTGTCATGACTGGAGTGATGAGCACTGCTTGAAATTTTTGAAGAAC 900
 901  TGCTATGAGGCACTGCCAGACAATGGAAAAGTGATTGTGGCAGAATGCATACTTCCAGTG 960
                                                  B
                                                  c
                                                  1
                                                  I
 961  GCTCCAGATTCAAGCCTGGCCAGAAAAGGTGTGTTCACATTGATGTGATCATGTTGGCT 1020
1021  CATAATCCTGGTGGGAAAGAGAGAACACAAAAAGAGTTTGAGGATCTTGCCAAAGGTGCT 1080
1080  GGATTCCAAGGTTTCAAAGTCCATTGTAATGCTTTCAACACATACATCATGGAGTTTCTT 1140
1141  AAGAAGGTTTAATTTCTTTGGTGTGTTGCATCTGAGTTTTGATATTGAGATTGTGGTTGT 1200
1201  GCTTCTACTTACCTAAGCTTTCCCCATAAAAATATGTGATTTCCACTTCTATTCGGTAGG 1260
1261  AAAATAATAATGAGAAAGTTCATTGTAATATTGCCTATATAAATGAACATTGTTTCATAT 1320
1321  TGTGGATTATAAAAAAAAAAA 1341  SEQ. ID NO. 1
```

FIGURE 7a

BamHI — COMT sequence starts from here

```
   1 ggatccccccgggctgcaggaattccAATCTCACAAAAACCTCATCAATCACAACCATGGGT  60
  61 TCAACAGGTGAAACTCAAATAACACCAACCCACATATCAGATGAAGAAGCAAACCTCTTC  120
 121 GCCATGCAACTAGCAAGTGCTTCAGTTCTTCCCATGATTTTGAAATCAGCTCTTGAACTT  180
 181 GATCTCTTAGAAATCATTGCTAAAGCTGGACCTGGTGCTCAAATTTCACCTATTGAAATT  240
 241 GCTTCTCAGCTACCAACAACTAACCCTGATGCACCAGTTATGTTGGACCGAATGTTCCGT  300
 301 CTCTTGGCTTGTTACATAATCCTCACATGTTCAGTTCGTACTCAACAAGATCGAAAGGTT  360
 361 CAGAGACTTTATGGTTTGGCTACTGTTGCTAAGTATTTGGTTAAGAATGAAGATCGTGTA  420
 421 TCCATTTCTGCTCTTAATCTCATGAATCAGGATAAACTGCTCATGCAAAGCTGGTACCAC  480
 481 CTAAAAGATGCAGTCCTTGATGGGGGCATTCCATTCAACAAGGCTTATGGAATGACAGCC  540
                                                              BclI
 541 TTTGAATACCATGGAACAGATCCAAGGTTTAACAAGCTTTTCAACAAGGGGATGTCTGAT  600
 601 CACTCTACCATCACAATGAAGAAAATTCTTGAGACCTACACAGGTTTTGAAGGCCTTAAA  660
 661 TCTCTTGTTGATGTAGGTGGTGGTACTGGAGCTGTAATTAACACGATTGTCTCAAAATAT  720
 721 CCCACTATAAAGGGTATAAATTTTGATTTACCCCATGTCATTGAAGATGCTCCATCTTAT  780
 781 CCAGGAGTTGAGCATGTTGGTGGAGACATGTTTGTCAGTATTCCAAAGGCTGATGCTGTT  840
 841 TTTATGAAGTGGATTTGTCATGACTGGAGTGATGAGCACTGCTTGAAATTTTTGAAGAAC  900
 901 TGCTATGAGGCACTGCCAGACAATGGAAAAGTGATTGTGGCAGAATGCATACTTCCAGTG  960
 961 GCTCCAGATTCAAGCCTGGCCACAAAAGGTGTGGTTCACATTGATGTGATCATGTTGGCT 1020
1021 CATAATCCTGGTGGGAAAGAGAGAACACAAAAAGAGTTTGAGGATCTTGCCAAAGGTGCT 1080
1080 GGATTCCAAGGTTTCAAAGTCCATTGTAATGCTTTCAACACATACATCATGGAGTTTCTT 1140
1141 AAGAAGGTTTAATTTCTTTGGTGTGTTGCATCTGAGTTTTGATATTGAGATTGTGGTTGT 1200
1201 GCTTCTACTTACCTAAGCTTTCCCCATAAAAATATGTGATTTCCACTTCTATTCGGTAGG 1260
1261 AAAATAATAATGAGAAAGTTCATTGTAATATTGCCTATATAAATGAACATTGTTTCATAT 1320
1321 TGTGGATTATAAAAAAAAAAA 1341  SEQ. ID NO. 1
```

FIGURE 7b

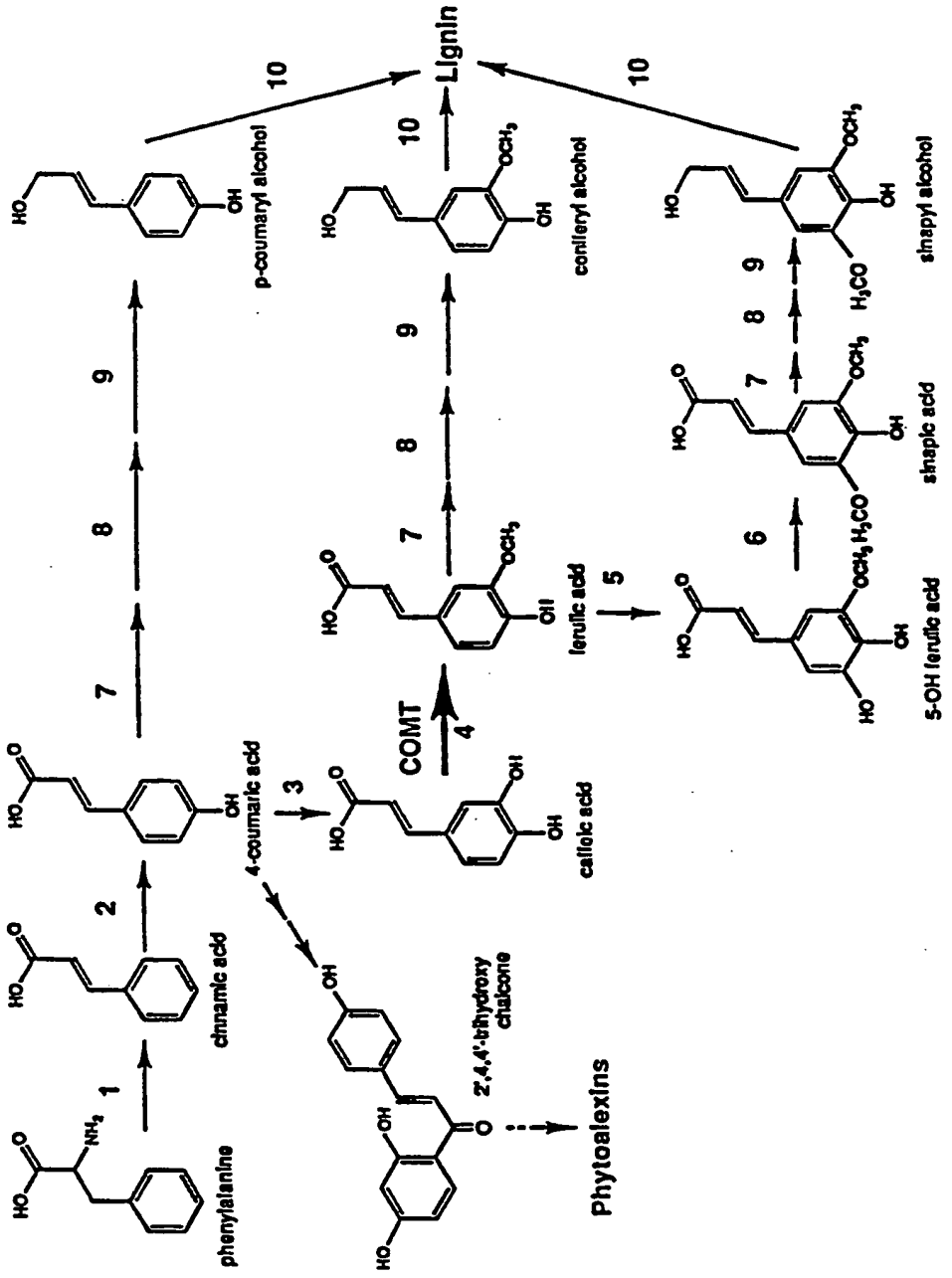
Lignin
10
10
10
p-coumaryl alcohol
coniferyl alcohol
sinapyl alcohol
9
8
7
6
5
4
3
2
1
COMT
ferulic acid
5-OH ferulic acid
shapic acid
caffeic acid
4-coumaric acid
cinnamic acid
phenylalanine
2',4,4'-trihydroxy chalcone
Phytoalexins

FIGURE 8

INTERNATIONAL SEARCH REPORT

Ir tional Application No
PCT/US 94/03356

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/82 C12N15/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. CELL. BIOCHEM. SUPPL., KEYSTONE SYMPOSIUM ON CROP IMPROVEMENT VIA BIOTECHNOLOGY : AN INTERNATIONAL PERSPECTIVE, HELD APRIL 10-16, 1992. vol. 16F , 1992 page 219 NI, W., ET AL. 'Modification of lignin biosynthesis by genetic manipulation of caffeic acid O-methyltransferase' see abstract Y219 ---	1-8
Y	WO,A,93 05160 (ICI) 18 March 1993 see the whole document ---	1-8
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 July 1994	- 1. 08. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	BIOLOGICAL ABSTRACTS /RRM ABSTRACT NO. BR46:67408 see abstract & J. CELL. BIOCHEM. SUPPL. vol. 18A , January 1994 page 96 WEITING, N., ET AL. 'Genetic manipulation of lignin biosynthesis'	1-8
A	PLANT PHYSIOLOGY vol. 97 , 1991 pages 7 - 14 GOWRI, G., ET AL. 'Stress responses in alfalfa (Medicago sativa L.) . X. Molecualr cloning and expression of S-adenosyl-L-methionine:caffeic acid 3-O-methyltransferase, a key enzyme of lignin biosynthesis' see the whole document	1-8
A	PLANT PHYSIOLOGY SUPPLEMENT vol. 99, no. 1 , May 1992 page 19 PODILA, G.K., ET AL. 'Antisense expression of an aspen O-methyltransferase construct in transgenic tobacco via Agrobacterium' see abstract 110	1-8
A	WO,A,93 05159 (ICI) 18 March 1993 see the whole document	1-8

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/US 94/03356

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9305160	18-03-93	AU-A-	2516792	05-04-93
		EP-A-	0603250	29-06-94
WO-A-9305159	18-03-93	AU-A-	1658192	05-04-93
		CA-A-	2109222	27-10-92
		EP-A-	0584117	02-03-94